Title: Transactivator protein: An alternative for delivery of recombinant proteins for safer reprogramming of induced Pluripotent Stem Cell

Authors: Fazlina Nordin, Raja Norazireen Raja Ahmad, Farzin Farzaneh

PII: S0168-1702(16)30834-6
DOI: http://dx.doi.org/doi:10.1016/j.virusres.2017.04.007
Reference: VIRUS 97115

To appear in: *Virus Research*

Received date: 19-12-2016
Accepted date: 8-4-2017

Please cite this article as: Nordin, Fazlina, Ahmad, Raja Norazireen Raja, Farzaneh, Farzin, Transactivator protein: An alternative for delivery of recombinant proteins for safer reprogramming of induced Pluripotent Stem Cell.Virus Research http://dx.doi.org/10.1016/j.virusres.2017.04.007

Transactivator protein: An alternative for delivery of recombinant proteins for safer reprogramming of induced Pluripotent Stem Cell

Fazlina Nordin[1], Raja Norazireen Raja Ahmad[1], and Farzin Farzaneh[2]

[1]Cell Therapy Centre (CTC), UniversitiKebangsaan Malaysia Medical Centre (UKMMC), Kuala Lumpur, Wilayah Persekutuan, 56000, Malaysia

[2]Immunology Group, Department of Molecular Medicine, King's College London, UK

Corresponding author: Fazlina Nordin (PhD), Research Fellow & Lecturer, Cell Therapy Centre UKMMC, 12th Floor, Clinical Block, UKM Medical Centre, JalanYaccob Latif, 56000 Cheras Kuala Lumpur, MALAYSIA; Email: nordinf@ppukm.ukm.edu.my

HIGHLIGHTS

- Definition and mechanisms of protein transduction delivery system based on HIV-TAT domain/peptide.
- Introduction of a novel TATκ (TAT_{kappa}), synthetic TAT peptides which was established by Professor Farzin Farzaneh from King's College London, UK.
- Discussion and comparison of the pioneer work using this novel TATκ by our group with most efficient used TAT33 peptide.
- The potential of using TAT domain as a tool delivery of transcription factors as an alternative for generating induced pluripotent stem cell (iPSC) for safer clinical applications.
- Modifications, evolution and future directions of using TAT peptide for efficient delivery of proteins/factors are also discussed.

Abstract

Induced pluripotent stem cells (iPSC) are somatic cells reprogrammed to pluripotency by forced expression of pluripotency factors. These cells are shown to have the same pluripotent potential as embryonic stem cells (ESC) and considered as an alternative to the much controversial usage of ESC which involved human embryos. However, the traditional method in reprogramming cells into iPSC using genome-integrating retro- or lenti- viruses remains an obstacle for its application in clinical setting. Although numerous studies have been conducted for a safer DNA-based reprogramming, reprogramming of iPSC by genetic modifications may raise the possibility of malignant transformation and has been a major limitation for its usage in clinical applications. Therefore, there is a need for an alternative method to reprogram the cells without the use of gene editing and a much safer way to deliver transcription factors to induce pluripotency on target cells. Using protein transduction approach, a number of studies have demonstrated the generation of human iPSCs from human fibroblasts and mouse embryonic fibroblasts by direct delivery of reprogramming proteins. In this review, the definition and mechanism of HIV-TAT protein (a type of protein transduction domain) in delivering recombinant proteins, including the potential of protein-based delivery to induce iPSC were further discussed.

Keywords: TAT protein; iPSC; reprogramming somatic cell; protein transduction.

Introduction

Since the revolutionary method of reprogramming somatic cells by retroviral vectors in 2006, (Takahashi and Yamanaka, 2006) subsequent studies later confirmed the generation of induced pluripotent stem cells (iPSCs) from human by forced expression using a panel of pluripotency factors (KLF4, Oct-3/4, Sox2 and c-Myc) (Wernig *et al*., 2007; Takahashi *et al*., 2007; Park *et al*., 2008). These iPSCs were indistinguishable from embryonic stem cells, with the ability to differentiate into various cell types. However, the use of genome-integrating retroviruses in these reprogramming methods, remain an obstacle for iPSC application in a clinical setting as it may cause unpredictable genetic dysfunction (Yamanaka 2007; Okita *et al*., 2007). Furthermore, a case of insertional mutagenesis in patient treated with retroviral mediated gene therapy limits the potential of iPSC generated from retrovirus delivery to be used in clinical settings (Hacein-Bey-Abina *et al*., 2003). To address these limitations, few strategies in silencing the transgenes in lentivirus delivery such as using inducible expression system (Brambrink *et al*., 2008; Soldner *et al*., 2009), has been reported. Other than viral delivery, transient transfection of piggyBac transposon with Cre-mediated excisable system has also been developed albeit with lower transfection efficiency (Kaji *et al*., 2009; Woltjen *et al*., 2009). Although all of these methods may provide a safer DNA-based reprogramming, integrative reprogramming could still induce genetic and epigenetic modification which resulted in somatic coding mutations (Gore *et al*., 2011).

Since then, many strategies to avoid random integration in reprogrammed cells has been explored through non-genetic manipulation techniques using Sendai virus (SeV) (Fusaki *et al.*, 2009), synthetic mRNA (Warren *et al*., 2010), mirRNA (Miyoshi *et al*., 2011) and episomal plasmid (Yu *et al*., 2009). In terms of reprogramming efficiencies, mRNA is reported to be higher than episomal and SeV but a survey throughout the reprogramming laboratory community around the world reveals 41% of the respondents could not achieved successful reprogramming by RNA method (Schlaeger *et al*., 2015). Thus, while highly efficient, RNA reprogramming proved to be challenging even in experienced laboratories. In terms of potential usage in clinical setting, episomal reprogramming method has been reported to be robust with higher reprogramming efficiencies than mRNA method in patient derived primary cell lines even under feeder free settings (Goh *et al*., 2013). However, among the limitations of episomal reprogramming are its low efficiency and elaborate combination of reprogramming factors other than the established four (Yu *et al.*, 2009). Recent study also reported that further optimisation of culture condition is needed to achieve a complete transgene free reprogramming using episomal plasmids (Li *et al*., 2016).

Another completely different approach towards transgene free cell reprogramming is through the usage of small molecules and chemicals. In 2008 paper, Huangfu *et al.,* has reported the use of histone deacetylase (HDAC) inhibitor, Valproic Acid (VPA) to improve reprogramming efficiencies (Huangfu *et al*., 2008). Since then, the usage of small molecules and chemicals to facilitate reprogramming through induction or by replacing one or two reprogramming factors have been widely explored (Lyssiotis *et al.*, 2009; Li *et al*,. 2009; Shi *et al.*, 2008). Small molecules and chemicals could modulate epigenetic mechanism and activate endogenous gene expressions through various signaling pathways in rapid, reversible and dose dependent manner (Huangfu *et al*., 2008; Mikkelsen *et al*., 2008). Although the usage of chemicals allows the ease of handling and administration, a comprehensive downstream screening is needed should it be used in clinical settings.

Protein-based delivery gained attention with the discovery that treatment of somatic cells with embryonic carcinoma (EC) and embryonic stem cell (ESC) cellular protein extracts could induced pluripotency and epigenetic reprogramming (Taranger *et al.,* 2005); Freberg et al., 2007). Both studies relied on the permeabilization of cell membranes to facilitate delivery of cell extracts into cells. Permeabilization of cell membrane is crucial as the absence of streptolysin-O (SLO) treatment hindered the up-regulation of pluripotency genes (Bru *et al.,* 2008). In 2010 paper, Cho *et al*., thoroughly explored the potential of protein-based reprogramming and reported a successful generation of human iPSC using only a single transfer of ESC-derived protein extracts (Cho *et al*., 2010). Hence, protein-based delivery is thought to be a potential alternative in reprogramming cells.

By using cell permeable protein transduction approach, protein could be delivered easily into cells without the use of permeabilization agent such as SLO (Ford *et al*., 2001). Protein transduction method was also shown to be having higher advantage than viral transgene delivery *in vivo* (Barka and Van der Noen, 1996; Barka *et al*., 2000). This delivery method ensures no genomic integration and is a practical reprogramming alternative in clinical settings. The protocol using recombinant proteins is straightforward, and there is certainly no need for integration-free colonies selection. Furthermore, protein reprogramming allows better timing and control of reprogramming factors delivery in a concentration-dependent manner. One concern that should be addressed is the relatively low efficiency of these protein-based reprogramming. However, with recent progress in this field, a complete exogene-free iPSC cell line could possibly be generated using a highly efficient protein-based reprogramming. In this review, we discuss the various types of protein transduction system, their mechanism, and

introduction to HIV-TAT derived transduction systems and its variations. In addition, the recent progress in protein-based delivery application in iPSC cell reprogramming is also reviewed.

Protein Transduction Technology

Protein transduction can be best described as the introduction of proteins into target cells from the external environment. Some proteins and peptides are capable of being taken up by cells or can penetrate the cell membrane, when introduced exogenously (Ford *et al.*, 2001). These proteins and peptides, usually either cytokines, homeoproteins or transcription factors, exhibit the unique properties of efficient translocation across cell membrane. This unique translocation is due to the presence of short amino acid sequences within the proteins, mediating cellular entry; which were identified as protein transduction domains (PTDs). PTDs are sometimes termed 'cell permeable proteins' (CPP) or 'membrane translocating sequences' (MTS) (Beerens *et al.*, 2003). This technology has opened new avenues to transfer proteins and other molecules into target cells for therapeutic purposes.

PTDs that chemically fuse to other proteins confer the ability to similarly penetrate the cell membrane and even the nucleus. The three most widely studied PTDs are the Drosophila antennapeptide (Antp), the herpes simplex virus VP22 protein, and the HIV TAT protein transduction motif (Ford *et al.*, 2001; Beerens *et al.*, 2003; Noguchi and Matsumoto, 2006b). These PTDs are different in terms of their amino acid sequence and length (Table 1) (Schwarze *et al.*, 2000). It has also been suggested that other proteins may have similar properties. These include fibroblast growth factor 1 and 2, and homeodomain (HD) containing proteins such as Hoxa-5, Hoxb-4, and Hoxc-8 (Prochiantz, 2000).

The reason why PTDs are widely used are: (i) they confer the ability to deliver macromolecules into target cells, (ii) they are mainly independent of cell types, (iii) they are often very efficient, (iv) they are mostly stable, (v) they have very low or no cytotoxicity, (vi) they allow precise time and dose management and (vii) they usually cause no genetic modification of target cells. These features have made PTDs a promising alternative to deliver therapeutic proteins in a safe manner.

The potential of TAT PTD to deliver therapeutic proteins for treatment of cancer has been widely studied. Although many studies have proven the uptake of TAT PTD fusion proteins by target cells, the trans-activation potential of endogenous cellular genes by the 11 amino acids remains unclear. In addition, the full-length expression of TAT protein stimulates the growth of Kaposi's sarcoma-derived cells (Ensoli *et al.*, 1990) and TAT transgenic mice develop Kaposi's sarcoma (Prakash *et al.*, 2000). However, the use of the 11 amino acids of TAT PTD is shown to be non-toxic *in vivo* experiments as this small PTD sequence is

responsible for protein translocation only (Beerens *et al.*, 2003). To date, no toxic effects or tumour transformed cells of abbreviated PTDs have been reported elsewhere.

Intracellular Delivery of PTD-Conjugated Macromolecules

Most studies have shown the ability of PTD fusion proteins to deliver a cargo of biologically active proteins (Barka and Van der Noen, 1996; Ho *et al.*, 2001; Noguchi *et al.*, 2004). However, the ability of PTDs to deliver a cargo into cells is not limited only to proteins or peptides. Other macromolecules including non-organic molecules fused or linked to different PTDs have also been shown to transduce into target cells efficiently. These macromolecules are antisense, short-interfering ribonucleic acids (siRNA), peptide nucleic acids (PNA), iron nanoparticles, liposomes, and plasmids (Prochiantz, 2000; Vives, 2003; Wadia and Dowdy, 2003; Noguchi and Matsumoto, 2006b) (Table 2).

Possible Mechanisms of Protein Transduction

The mechanism of the internalization of PTDs is poorly understood. A few studies have shown that the internalization of TAT peptide is energy-dependent, requiring a temperature above 4 ºC and ATP (Brooks *et al.*, 2005). Nevertheless, many studies have demonstrated that the internalization of TAT-PTD, Antennapedia (Antp)-PTD or poly-Arginine (polyR)-PTD is not significantly inhibited by incubation at 4ºC, by inhibitors of endocytosis or by depletion of cellular ATP (Futaki *et al*., 2001). Also demonstrated is that PTD internalization does not depend on a specific primary sequence; hence, it is receptor-independent (Vives *et al*., 1997). A few models have emerged to explain PTD uptake into target cells by the perpendicular insertion of amphipathic peptides, electrostatic interaction or endocytosis.

The first model is the perpendicular insertion of amphiphatic peptides into the membrane (Noguchi and Matsumoto, 2006b). This mechanism involves assembled hydrophilic-hydrophobic lipids forming a channel through oligomerization, allowing efficient passage of hydrophilic cargo. However, several peptides can form pores at the cell surface, thus would be a threat to cell survival by inducing apoptosis. Antp-PTD may be categorized in this model due to the presence of penetratin, which is derived from the third helix of the Antphomeodomain protein (position 43-58 amino acids) (Prochiantz, 2000). This peptide contains tryptophan (a hydrophobic amino acid) which is reported to be critical for the carrier ability in this model (Prochiantz, 2000).

A second model proposes electrostatic interaction between peptides binding to the polar heads of lipids in the membrane, resulting in the formation of inverted micelles that engulf the cargo. Interestingly, this process occurs even at 4ºC, thus confirming that the mechanism is temperature-independent. The micelle that was formed earlier will then re-open either in the

cytoplasm or nuclei of target cells. Some studies suggest that Antp-PTD might use this pathway (Berlose *et al.*, 1996). It has also been suggested that HIV-TAT, which shares similar charge characteristics may also use this mechanism (Ford *et al.*, 2001). However, a drawback of this model is that the inverted micelle formation would limit cargo size and it also requires the presence of hydrophobic amino acids, such features are absent in TAT PTD and poly-arginine-PTDs.

A third model proposes cargo uptake by macropinocytosis, a type of endocytosis. It involves three phases which are: an electrostatic interaction with plasma membrane, macropinocytosis and retrograde transport (Figure 1). Most PTD-containing compounds are highly positively charged, while the plasma membrane is always negatively charged, thereby promoting PTD binding to plasma membrane due to electrostatic interaction. The initial step of transduction by PTDs occurs with electrostatic interaction with the plasma membrane, followed by the penetration into cells by macropinocytosis and release of the protein into cytoplasm and nuclei by retrograde transport to the Golgi apparatus and endoplasmic reticulum (Lord and Roberts, 1998; Noguchi and Matsumoto, 2006a). Macropinocytosis is the invagination of the membrane forming a pocket which then forms a vesicle filled with extracellular matrix (including molecules within it). The endocytosis pathway appears to be critically important for the internalization of PTD-containing compounds.

HIV TAT Protein Transduction Domain

The HIV-1 *trans*-activator gene product, TAT, has been shown to be a regulator of transcription in latent HIV and essential for HIV replication (Karn, 2011). It is an 86 amino acid protein consisting of two exons of 72 and 14 amino acids respectively. It has been reported that HIV-TAT added exogenously in culture media was taken up efficiently by target cells (Green and Loewenstein, 1988). TAT amino acids 38-58 (the basic region of TAT) retain the transducing ability of HIV-TAT, which enables both nuclear and cytoplasmic uptake of the proteins (Mann and Frankel, 1991). This study also showed that the uptake was not inhibited by trypsinazation, heparinazation and neuramidazation of the cells. Furthermore, they speculated that TAT binding to the cellular membrane was mediated through charge-interaction between the basic region of TAT and charged polysaccharides on the cellular membrane of the target cells (Mann and Frankel, 1991).

In subsequent studies, Green and Loewenstein (1998) have shown that the improved TAT (YGRKKRRQRRR) fusion proteins transduced better into target cells. This improved synthetic TAT domain corresponds to a short 11 amino acid region which is highly positively charged at physiological pH with nine out of 11 of its amino acids being either Arginine or

lysine. Since then, a series of synthetic TAT PTDs have been produced by optimising the deletion, addition and replacement of Arginine residues of primary TAT sequences, in order to determine their structure, activity relationship and the effect on internalization efficiency (Wender *et al.*, 2000; Ho *et al.*, 2001).

A few studies have demonstrated the ability of synthetic HIV-TAT to deliver several peptides and proteins into target cells. This includes poly-Arginine (polyR) and poly-lysine, which exhibit even greater delivery efficiency compared to the primary sequence of the original TAT (Futaki *et al.*, 2001; Noguchi *et al.*, 2004). Ho *et al.,* (2001) reported the establishment of the improved synthetic TAT domains by α-helix modification of the original TAT structure (Figure 2) (Ho *et al.*, 2001). These synthetic TAT domains were named as PTD-3, PTD-4, PTD-5, PTD-6, PTD-7 and PTD-8. The transduction efficiency was increased 5-fold in both PTD-3 and PTD-6, 8-fold in PTD-5 and 33-fold in PTD-4. However, the transduction efficiency of PTD-7 and PTD-8 peptides containing nine or seven Arginine residues was comparable with the original TAT peptide. In this review, the most studied peptide, PTD-4 will be further discussed together with the novel TATκ, established by Flinterman and colleagues in 2009 (Flinterman *et al.*, 2009).

PTD-4 (also known as TAT33)

PTD-4 was derived from the original TAT peptide by strengthening the putative *α-helix* with Ala (Alanine) residues (Ho *et al.*, 2001). The Arg (Arginine) content of TAT33 was limited to three, which were closely aligned down the face of the α-helix structure (Figure 2C). This was done by synthesizing the original TAT peptide with an NH2-terminal FITC-Gly (Glycine) residue followed by a Gly-Gly-Gly motif (Glycine). The Ala residues were then placed accordingly at the appointed positions (2, 4, 5, 6, 9 and 11). This synthetic TAT33 showed dramatically increased transduction efficiency (up to 33-fold) as compared to the original TAT peptide. The differences in the amino acid sequence between TAT-33 and TAT are shown in Figure 3.

A study by Mi *et al.,* (2000) demonstrated that the biotinlyted TAT33 and TAT peptides fused to avidin-beta-galactosidase were efficiently transduced in a variety of cell lines and primary cells (Mi *et al.*, 2000). However, the transduction efficiency of TAT33 was comparable with TAT peptides. Another study also demonstrated that mice injected with either TAT33 or TAT peptides had about ~100% of their blood cells transduced 30 minutes post-intravenous injection. Additionally, FACS analysis showed 5-fold intracellular PTD-4-FITC accumulation compared to TAT peptide (Ho *et al.*, 2001).

TAT_{kappa} (TATκ)

TATκ was generated by introducing mutations to destroy the two furin cleavage sites that were present within the original TAT peptide (Figure 3). Furin is a human gene also known as PACE (*Paired basic Amino acid Cleaving Enzyme*) which belongs to the subtilisin-like proproteinconvertase family. Furin is enriched in the Golgi apparatus but can translocate between the trans-Golgi network and the cell surface (Denault and Leduc, 1996). The presence of these Furin sites in the TAT peptide results in the TAT peptide being cleaved from fusion proteins that are secreted via the constitutive pathway (Flinterman *et al.*, 2009). Thus, the secreted protein will not be able to enter target cells and is trapped in the culture supernatant. Therefore, amino acid sequences RQRR and RKKR of TAT were modified by replacing five Ala residues within the peptide.

Flinterman *et al.,* (2009) have established a novel system for the secretion of TAT fusion proteins using a mammalian expression system. Moreover, the ability of the secreted TATκ-GFP to efficiently transduce target cells was far greater than could be achieved with TAT-GFP or GFP alone. They also showed that the improved TATκ-GFP was efficiently secreted in culture supernatant compared to TAT-GFP confirming the presence of furin sites within the original TAT PTD. The secreted TATκ-GFP fused to Apoptin was also able to transduce into human osteosarcoma cell line (Saos-2) and induce apoptosis (Flinterman *et al.*, 2009).

PTD usage in directing iPSC reprogramming

Using protein transduction approach, Zhou *et al.,* (2009) first showed generation of mouse iPS cells by transduction of purified 11 Arginine (11R)-tagged recombinant protein combined with chemical molecules such as valproic acid (VPA) (Zhou *et al.*, 2009). The mouse iPS cells were able to be expanded more than 30 passages, generate three primary germ layers and give rise to chimeric mice. Subsequently, *Kim et al.,* (2009) reported successful iPSC generation in human newborn fibroblast (HNF) cells using multiple treatments of cell extracts from stable HEK293 cell lines expressing four reprogramming factors Oct-4, Klf4, Sox2, c-Myc (OKSM) fused to 9 Arginine (9R). Encouragingly, these human iPSC were also able to differentiate into functional dopamine (DA) neurons and could significantly rescue motor deficits in Parkinson Disease (PD) rat model (Rhee *et al*., 2011). In a 2012 paper, Zhang *et al.,* (2012) described the comparison between the usage of 11R and TAT as protein transduction method in generating iPSC. It was found that recombinant proteins fused with TAT (TAT-RF) has a higher transcriptional activity compared to 11R-recombinant protein (11R-RF) suggesting TAT as the better choice as PTD for protein-based delivery (Zhang *et al*., 2012). The authors also reported higher efficiencies (0.012%) of iPSC reprogramming using five factors TAT-RF (with the addition of Nanog) combined with VPA treatment as compared to the previous studies (Zhou *et al*., 2009; Kim *et al*., 2009). In a later study, Nemes *et al*., (2014) employed a strategy using

transduction of GST-tagged TAT recombinant protein with a nuclear localization signal polypeptide (NLS) to further facilitate nuclear localization and successfully produced iPS cell lines which were able to incorporate into the blastocyst and generate chimeric offspring in mice (Nemes *et al*., 2014). In terms of functionality of the recombinant reprogramming proteins, Thier *et al.,* has established a lipid-rich albumin supplement in a low serum culture conditions for protein delivery of TAT-Oct-4, and TAT-Sox2. Both recombinant proteins are biologically active and able to be a substitute for their viral counterpart and in inducing iPSC reprogramming (Thier *et al*., 2010; Thier *et al*., 2012).

Limitations and possible ways to overcome

Although several studies have reported successful generation of iPSC (Zhou *et al*., 2009; Kim *et al*., 2009; Zhang *et al*., 2012; Nemes *et al*., 2014) the efficiency of iPSC generation, using protein transduction approach is significantly lower (about 0.001% of input cells) compared to viral transduction approach (about 0.01% of input cells) (Takahashi *et al.*, 2007; Park *et al.*, 2008; Kim *et al.*, 2009). In an effort to improvised reprogramming protocols using PTD, Lee *et al*., 2012 observed significant differences in the genes expression profiles of cell reprogrammed with retrovirus and protein transduction method. It was reported that a toll-like receptor 3 (TLR3) pathway which enables efficient pluripotency reprogramming was activated only in retroviral induced cells as opposed to protein-based delivery (Lee *et al*., 2012). The study concluded that innate immunogenicity induced by viral particles could activate the expression of inflammatory genes and enhance epigenetics regulation in favor of reprogramming. However, application of TLR agonists called polyinosinic-polycytidylic acid (Poly I: C) together with recombinant proteins delivery could induce reprogramming efficiency in a shorter time (Lee *et al*., 2012). This strategy of using identified small molecules in enhancing the reprogramming efficiencies by recombinant proteins delivery method have also been employed in other studies with encouraging results (Zhou *et al*., 2009; Zhang *et al*., 2012). Furthermore, the use of other small molecules such as butyrate, 8-bromoadenosine and thiazovivin in enhancing reprogramming efficiencies in other integrative and non-integrative protocols have also been extensively reported (Mali *et al.*, 2010; Lin *et al*., 2009; Wang and Adjaye, 2011). Thus, further study is needed to explore the potentials of other small chemicals in supporting protein-based reprogramming as a way to overcome its limited reprogramming capabilities.

Another concern in direct delivery of recombinant factors into cells is the production and quality of recombinant proteins. TAT recombinant proteins could be produced using bacterial expression vectors in a highly efficient manner (Nagahara *et al*., 1998) however subsequent purification steps may caused recombinant proteins to be insoluble, denatured

and could affect its biological activity (Singh *et al*., 2015). Although numerous studies have shown that TAT and 9R denatured proteins could be refolded and regained its biological activity inside the mammalian cells (Kwon *et al*., 2000; Jin *et al*, 2001), denatured proteins were also thought to be one of the main cause of unsuccessful reprogramming in human somatic cells (Pan *et al.*, 2010). As the quality of recombinant protein produced in mammalian cells is superior than the one produced in bacteria or yeast and largely employed in most pharmaceutical proteins (Wurm *et al.*, 2004), mammalian cells expression system could be a potential alternative in the production of correctly folded protein with a functional post translational modification in reprogramming applications (Hartley, 2012) .

To date there has been only one study employing mammalian cells HEK293 for expressing the OSKM 9R-recombinant proteins and its subsequent direct application of cells extract to target cells (Kim *et al*., 2009). The authors observed that a single 16 hour exposure of protein treatment were not sufficient and concluded that continuous exposure to reprogramming factors is needed. They were only able to induce reprogramming in HNF through a multiple round of 16 hour protein treatments followed by 6 days incubation in hESC culture medium (Kim *et al*., 2009). This method while feasible could be laborious as multiple rounds of transduction is needed in a span of 8 weeks. As an alternative, we recently described a strategy to develop producer cell lines that could continuously express reprogramming factors for applications in the targeted cell lines through direct or indirect co-culture. Using a modified TAT protein TATκ, we have shown in our recent study a strategy in generating 293T cells secreting the pluripotent factors Oct-3/4 (Nordin *et al.,* 2014). The luciferase assay analysis showed a weak biological activity of Oct-3/4 protein, which confirmed that these producer cells secreted TATκ fused to Oct-3/4 protein in culture medium and could be uptake by haematopoietic cell lines, Jurkat and FDCP-1 cells. Although there are limitations that needed to be addressed, this strategy using cell lines in expressing recombinant proteins could be potentially applied in the future for the generation of iPS cells by protein transduction.

As an alternative to the perceived cumbersome approach in reprogramming using recombinant proteins, few studies have identified the feasibility of using self-penetrating proteins as an alternative approach. Native reprogramming Oct-4 protein were reported to be having self-penetrating capability and are able to be uptake into living cells as well as translocated to the nucleus (Harretheir *et al*., 2014). Other studies also reported the ability of various versions of Nanog and Sox2 proteins in retaining and promoting their reprogramming capabilities (Theunissen *et al*., 2011; Aksoy *et al*., 2013). Thus, with the use of reprogramming factor proteins either truncated or enhanced with transactivator domains, protein-based delivery method is still in the running towards the race in generating clinically safer iPSC.

Future directions

When taken together with previous reports, protein transduction method is a feasible alternative in replacing integration-based reprogramming methods. However, more basic questions such as immunogenicity, protein half-life, protein concentration, and modes of delivery are important questions that need to be answered before this type of therapy can be considered for long-term and effective human applications. At the molecular level, it is unclear how proteins behave when interacting with the cell membrane to mediate entry. It is thought that protein transduction across the cellular membrane resulted in partial or complete unfolding of the protein which may differ from one protein to another (Schwarze *et al*., 2000). In order to obtain biologically functional protein, the transduced protein requires refolding once inside the target cells (Bonifaci *et al*., 1995). Another consideration is to investigate phenotypic changes to determine how promising *in vivo* protein transduction really is by conducting animal studies.

Recently, a growing number of studies have shown the feasibility of protein transduction method in replacing viral delivery methods for directing specific lineage differentiation. Direct lineage reprogramming gained much attention recently as a more feasible way to produce specific functional cell lines for usage in regenerative medicine (Xu *et al*., 2015). Specific lineage differentiation is mainly achieved through forced expression of known combinations of transcription factors specific to the cells such as cardiomyocyte (Ieda *et al*., 2010), neural stem cells (Han *et al*., 2012) and insulin-producing cells (Xu *et al*., 2013). Islas *et al.,* (2012) first attempted to reprogram human dermal fibroblast into cardiac progenitor using recombinant TAT fused with MESP1 and ETS2 and obtained cardiac progenitor cells (Islas *et al*., 2012).). Dai *et al.,* (2014) used recombinant TAT proteins fused to OSK factors (Oct-4, Sox2, Klf4) combined with small molecules to reprogrammed adipose derived stem cells into corneal-endothelial (CE) like cells (Dai *et al*., 2014). In a comparison study, myogenic transcription factors, MyoD tagged with TAT protein (TAT-MyoD) were shown to have higher reprogramming efficiency than its wild type MyoD protein which is also capable to facilitate transduction in mouse primary cells (Hidema *et al*., 2011).

Aside from HIV-TAT usage in protein transduction, several other studies reported the use of other protein-based delivery in directing differentiation. Transduction of Pdx1 and NeuroD protein which have intrinsic PTD sequences with the combination of MafA fused with 11R were reported to induce differentiation of insulin-producing cells in mouse ES cells and mouse iPS cells (Kaitsuka *et al*., 2014). Hu *et al.,* (2014) reported the application of C-end Rule (CendR) peptide to deliver transcription factor Sox2 to retinal pigmented epithelial cell (RPE) for reprogramming of functional neurons (Hu *et al*., 2014). Recently, one study applied QQ

reagent which allowed efficient protein transduction (Li *et al*., 2008) modified with Gata4, Hand2, Mef2c and Tbx5 with the combination of growth factors could induce 80% of HDF into cardiac progenitor cells and improved cardiac function after myocardial infarction in rat model (Li *et al*., 2015). Given these recent developments, protein transduction method has a great potential and future in directing lineage specific differentiation *in vitro* and *in vivo*, and subsequently could potentially be used in therapeutic application.

In general, a few improvements could be explored in the near future; (i) identifying new potential *protein transduction domains* (PTDs) and improving the potential of existing PTDs to increase transduction into target cells, (ii) identifying potential chemical molecules that are non-toxic to enhance transduction into target cells, (iii) to increase the refolding rates of transduced full-length proteins and domains, and (iv) to produce sufficient physiological levels of secreted proteins for large scale studies. As regards to the usage of HIV-TAT as PTD, more optimization needs to be performed in order to improve the protein transduction of pluripotency factors fused to TAT domain. As reported in our study, TATκ fusion with pluripotent factors could be potential method for directing reprogramming of cells (Nordin *et al.,* 2014). However there is a need to identify the problems related to secretion of TATκ fusion proteins, to study the biological function of TATκ fusion proteins in target cells and, to establish *in vitro* functional assays for assessment of these fusion proteins.

Therefore, understanding and improving the mechanisms involved in the direct delivery of PTD fusion proteins into target cells for iPS reprogramming could give the answer to these questions and allow an accurate assessment of the potential that this approach could offer for human therapeutic applications.

In conclusion, the protein transduction system has several advantages over integrating vectors; (i) they effectively eliminate the potential risks associated with chromosomal integrations, and (ii) they are a simpler and faster approach without the need for sequential selection of integration-free transduced cells, and (iii) the continuous expression of pluripotency factors by producer cell line which can be withdrawn at any time point in culture as compared to permanent gene expression in integrating viral vector. In addition, the assumption that a protein transduction approach, as opposed to genetic modification avoids the risk of malignant transformation requires extensive investigation to be verified by both *in vitro* and *in vivo* studies.

Conflict of interest:

The authors declare no conflict of interest.

References

Aksoy, I., Jauch, R., Eras, V.,Chng, W.B., Chen, J., Divakar, U., Ng, C.K, Kolatkar, P.R., and Stanton, L.W. (2013). Sox transcription factors require selective interactions with Oct4 and specific transactivation functions to mediate reprogramming. Stem Cells *31,* 2632-46.

Barka, T., and Van der Noen, H.M. (1996). Retrovirus-mediated gene transfer into salivary glands in vivo. Hum Gene Ther *7*, 613-618.

Barka, T., Gresik, E.W., and van Der Noen, H. (2000). Transduction of TAT-HA-beta-galactosidase fusion protein into salivary gland-derived cells and organ cultures of the developing gland, and into rat submandibular gland in vivo. J Histochem Cytochem *48*, 1453-1460.

Beerens, A.M., Al Hadithy, A.F., Rots, M.G., and Haisma, H.J. (2003). Protein transduction domains and their utility in gene therapy. Curr Gene Ther *3*, 486-494.

Berlose, J.P., Convert, O., Derossi, D., Brunissen, A., and Chassaing, G. (1996). Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments. Eur J Biochem *242*, 372-386.

Bonifaci, N., Sitia, R., and Rubartelli, A. (1995). Nuclear translocation of an exogenous fusion protein containing HIV Tat requires unfolding. AIDS *9*, 995-1000.

Brambrink, T., Foreman, R., Welstead, G.G., Lengner, C.J., Wernig, M., Suh, H., and Jaenisch, R. (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell *2*, 151-159.

Brooks, H., Lebleu, B., and Vives, E. (2005). Tat peptide-mediated cellular delivery: back to basics. Adv Drug Deliv Rev *57*, 559-577.

Bru, T., Clarke, C., McGrew, M.J., Sang, H.M., Wilmut, I., and Blow, J.J. (2008). Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts. (2008). Exp. Cell. Res. *314*, 2634-42.

Cho, H.J., Lee, C.S., Kwon, Y.W., Paek, J.S., Lee, S.H., Hur, J., Lee, E.J., Roh, T.Y., Chu, I.S., Leem, S.H., Kim, Y., Kang, H.J., Park, Y.B., and Kim, H.S. (2010). Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. Blood *116*, 386-395.

Dai, Y., Guo, Y., Wang, C., Liu, Q., Yang, Y., Li, S., Guo, X., Lian, R., Yu, R., Liu, H., and Chen, J. (2014). Non-genetic direct reprogramming and biomimetic platforms in a preliminary study for adipose-derived stem cells into corneal endothelia-like cells. PLoS One *9*, e109856.

Denault, J.B., and Leduc, R. (1996). Furin/PACE/SPC1: a convertase involved in exocytic and endocytic processing of precursor proteins. FEBS Lett *379*, 113-116.

Ensoli, B., Barillari, G., Salahuddin, S.Z., Gallo, R.C., and Wong-Staal, F. (1990). Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature *345*, 84-86.

Flinterman, M., Farzaneh, F., Habib, N., Malik, F., Gaken, J., and Tavassoli, M. (2009). Delivery of therapeutic proteins as secretable TAT fusion products. Mol Ther *17*, 334-342.

Ford, K.G., Souberbielle, B.E., Darling, D., and Farzaneh, F. (2001). Protein transduction: an alternative to genetic intervention? Gene Ther *8*, 1-4.

Freberg, C.T., Dahl, J.A., Timoskainen, S., and Collas, P. (2007). Epigenetic reprogramming of OCT4 and NANOG regulatory regions by embryonal carcinoma cell extract. Mol. Biol. Cell. *18*, 1543-53.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K. and Hasegawa, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci. *85*, 348-362.

Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001). Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J Biol Chem *276*, 5836-5840.

Goh, P.A., Caxaria, S., Casper, C., Rosales, C., Warner, T.T., Coffey, P.J., and Nathwani, A.C. (2013). A systematic evaluation of integration free reprogramming method for deriving clinically relevant patient specific induced pluripotent stem cells (iPS) cells. PLoS One *26*, e81622.

Gore, A., Li, Z., Fung, H.L., Young, J.E., Agarwal, S., Antosiewicz-Bourget, J., Canto, I., Giorgetti, A., Israel, M.A., Kiskinis, E., Lee, J,H., Loh, Y.H., Manos, P.D., Montserrat, N., Panopoulos, A.D., Ruiz, S., Wilbert, M.L., Yu, J., Kirkness, E.F., Izpisua Belmonte, J.C., Rossi, D.J., Thomson, J.A., Eggan, K., Daley, G.Q., Goldstein, L.S., and Zhang, K. (2011). Somatic coding mutations in human induced pluripotent stem cells. Nature *471*, 63-7.

Green, M., and Loewenstein, P.M. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell *55*, 1179-1188.

Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M., and Fischer, A. (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med *348*, 255-6.

Han, D.W., Tapia, N., Hermann, A., Hemmer, K., Hoing, S., Arauzo-Bravo, M.J., Zaehres, H., Wu, G., Frank, S., Moritz, S., Greber, B., Yang, J.H., Lee, H.T., Schwamborn, J.C., Storch, A., and Scholer, H.R. (2012). Direct reprogramming of fibroblasts into neural stem cells by defined factors. Cell Stem Cell *10*, 465-72.

Harreither, E., Rydberg, H.A., Amand, H.L., Jadhav, V., Fliedl, L., Benda, C., Esteban, M.A., Pei, D., Borth, N., Grillari-Voglauer, R., Hommerding, O., Edenhofer, F., Norden, B., and Grillari, J. (2014). Characterization of a novel cell penetrating peptide derived from human Oct4. Cell Regen (Lond) *31*, 2.

Hartley, J.L. (2012). Why proteins in mammalian cells?. Methods Mol Biol *801*, 1-12.

Hidema, S., Tonomura, Y., Date, S., and Nishimori, K. (2011). Effects of protein transduction with intact myogenic transcription factors tagged with HIV-1 Tat-PTD (T-PTD) on myogenic differentiation of mouse primary cells. J Biosci Bioeng *113*, 5-11.

Ho, A., Schwarze, S.R., Mermelstein, S.J., Waksman, G., and Dowdy, S.F. (2001). Synthetic protein transduction domains: enhanced transduction potential in vitro and in vivo. Cancer Res *61*, 474-477.

Hu, Q., Chen, R., Teesalu, T., Ruoslahti, E., and Clegg, D.O. (2014). Reprogramming human retinal pigmented epithelial cells to neurons using recombinant proteins. Stem Cells Transl Med *3*, 1526-34.

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A.E., and Melton, D.A. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol *26*, 795-797.

Ieda, M., Fu, J.D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G., and Srivasta, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell *142*, 375-86.

Islas, J.F., Liu, Y., Weng, K.C., Robertson, M.J., Zhang, S., Prejusa, A., Harger, J., Tikhomirova, D., Chopra, M., Iyer, D., Mercola, M., Oshima, R.G., Willerson, J.T., Potaman, V.N., and Schwartz, R.J. (2012). Transcription factors ETS2 and MESP1 transdifferentiate human dermal fibroblasts into cardiac progenitors. Proc Natl Acad Sci U.S.A *109*, 13016-21.

Jin, L.H., Bahn, J.H., Eum, W.S., Kwon, H.Y., Jang, S.H., Han, K.H., Kang, T.C., Won, M.H., Kang, J.H., Cho, S.W., Park, J., and Choi, S.Y. (2001). Transduction of human catalase mediated by an HIV-1 TAT protein basic domain and arginine-rich peptides into mammalian cells. Free Radic Biol Med *31*, 1509-19.

Kaitsuka, T., Noguchi, H., Shiraki, N., Kubo, T., Wei, FY., Hakim, F., Kume, S., and Tomizawa, K. (2014). Generation of functional insulin-producing cells from mouse embryonic stem cells through 804G cell-derived extracelullar matrix and protein transduction of transcription factors. Stem Cells Transl Med *3*, 114-27.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature *458*, 771-775.

Karn, J. (2011). The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. Curr Opin HIV AIDS 6 (*1)*: 4-11.

Kim, D., Kim, C.-H., Moon, J.-I., Chung, Y.-G., Chang, M.-Y., Han, B.-S., Ko, S., Yang, E., Cha, K.Y., Lanza, R., and Kim, K.-S. (2009). Generation of Human Induced Pluripotent Stem Cells by Direct Delivery of Reprogramming Proteins. Cell Stem Cell *4*, 472-476.

Krosl, J., Austin, P., Beslu, N., Kroon, E., Humphries, R.K., and Sauvageau, G. (2003). In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. Nat Med *9*, 1428-1432.

Kwon, H.Y., Eum, W.S., Jang, H,W., Kang, J.H., Ryu, J., Ryong Lee, B., Jin, L.H., Park, J., and Choi, S.Y. (2000). Transduction of Cu, Zn-superoxide dismutase mediated by an HIV-Tat protein basic domain into mammalian cells. FEBS Lett *485*, 163-7.

Lee, J., Sayed, N., Hunter, A., Au, K.F., Wong, W.H., Mocarski, E.S., Pera, R.R., Yakubov, E., and Cooke, J.P. (2012). Activaion of innate immunity is required for efficient nuclear reprogramming. Cell *151*, 547-558.

Li, Q., Huang, Y., Xiao, N., Murray, V., Chen., J., and Wang, J. (2008). Real time investigation of protein folding, structure, and dynamics in living cells. Methods Cell Biol *90*, 287-325.

Li, S., Lan, H., Men, H., Wu, Y., Li, N., Capecchi, M.R., Bryda, E.C and Wu, S. (2016). Derivation of transgen-free rat induced pluripotent stem cells approximating the quality of embryonic stem cells. Stem Cells Transl Med *6 (2)*: 340-351.

Li, W., and Ding, S. (2009). Small molecule that modulate embryonic stem cell fate and somatic cell reprogramming. Trends Pharmacol Sci *31*, 36-45.

Li, X.H., Li, Q., Jiang, L., Deng, C., Liu, Z., Fu, Y., Zhang, M., Tan, H., Feng, Y., Shan, Z., Wang, J., and Yu, X.Y. (2015). Generation of functional human cardiac progenitor cells by high-efficiency protein transduction. Stem Cells Transl Med *4*, 1415-24.

Lim, J., Kim, J., Kang, J., and Jo, D (2014). Partial somatic to stem cell transformations induced by cell-permeable reprogramming factors. Scientific Reports *4*: 4361.

Lin, T., Ambasudhan, R., Yuan, X., Li, W., Hilcove, S., Abujarour, R., Lin, X., Hahm, H.S., Hao, E., Hayek, A., and Ding, S. (2009). A chemical platform for improved induction of human iPSCs. Nat Methods *6*, 805-808.

Lord, J.M., and Roberts, L.M. (1998). Toxin entry: retrograde transport through the secretory pathway. J Cell Biol *140*, 733-736.

Lyssiotis, C.A., Foreman, R.K., Staerk, J., Garcia, M., Mathur, D., Markoulaki, S., Hanna, J., Lairson, L.L., Charette, B.D., Bouchez, L.C., Bollong, M., Kunick, C., Brinker, A., Cho, C.Y., Schultz, P.G. and Jaenisch, R. (2009). Reprogramming if murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4. Proc Natl Acad Sci U.S.A *106*, 8912-7.

Mali, P., Chou, B.K., Yen, J., Ye, Z., Zou, J., Dowey, S., Brodsky, R.A., Ohm, J.E., Yu, W., Baylin, S.B., Yusa, K., Bradley, A., Meyers, D.J., Mukherjee, C., Cole, P.A and Cheng, L. (2010). Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodelling and the expression of pluripotency-associated genes. Stem Cells *28*, 713-20.

Mann, D.A., and Frankel, A.D. (1991). Endocytosis and targeting of exogenous HIV-1 Tat protein. EMBO J *10*, 1733-1739.

Mi, Z., Mai, J., Lu, X., and Robbins, P.D. (2000). Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo. Mol Ther *2*, 339-347.

Mikkelsen, T.S, Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, S., and Mesissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature *454*, 49-55.

Miyoshi, N., Ishii, H., Nagano, H., Haraguchi, N., Dewi, D.L., Kano, Y., Nishikawa, S., Tanemura, M., Mimori, K., Tanaka, F., Saito, T., Nishimura, J., Takemasa, I., Mizushima, T., Ikeda, M., Yamamoto, H., Sekimoto, M., Doki., Y and Mori, M. (2011). Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell *8*, 633-8.

Nagahara, H., Vocero-Akbani, A.M., Snyder, E.L., Ho, A., Latham, D.G., Lissy, N.A., Becker-Hapak, M., Ezhevsky, S.A., and Dowdy S.F. (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Med *4*, 1449-52.

Nemes, C., Varga, E., Polgar, Z., Klincumhom, N., Pirity, M.K., and Dinyes, A. (2014). Generation of mouse induced pluripotent stem cells by protein transduction. Tissue Eng Part C Methods *20*, 383-92.

Noguchi, H., and Matsumoto, S. (2006a). Protein transduction technology offers a novel therapeutic approach for diabetes. J Hepatobiliary Pancreat Surg *13*, 306-313.

Noguchi, H., and Matsumoto, S. (2006b). Protein transduction technology: a novel therapeutic perspective. Acta Med Okayama *60*, 1-11.

Noguchi, H., Matsushita, M., Okitsu, T., Moriwaki, A., Tomizawa, K., Kang, S., Li, S.T., Kobayashi, N., Matsumoto, S., Tanaka, K., Tanaka, N., and Matsui, H. (2004). A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. Nat Med *10*, 305-309.

Nordin, F., Tye, G.J, Gaken, J., and Farzaneh, F. (2014). TATκ fusion protein of OCT-3/4 and KLF-4: Stable mixed population cell lines capable of delivering fusion proteins to target cells. J Cell Sci Ther 5(*2*):158.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313-7.

Pan, C., Lu, B., Chen, H., and Bishop, C. (2010). Reprogramming human fibroblasts using HIV-1 TAT recombinant proteins OCT4, SOX2, KLF4 and c-MYC. Molecular Biology Reports *37*, 2117-2124.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley, G.Q. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. Nature *451*, 141-146.

Prakash, O., Tang, Z.Y., He, Y.E., Ali, M.S., Coleman, R., Gill, J., Farr, G., and Samaniego, F. (2000). Human Kaposi's sarcoma cell-mediated tumorigenesis in human immunodeficiency type 1 tat-expressing transgenic mice. J Natl Cancer Inst *92*, 721-728.

Prochiantz, A. (2000). Messenger proteins: homeoproteins, TAT and others. Curr Opin Cell Biol *12*, 400-406.

Rhee, Y.H., Ko, J.Y., Chang, M.Y., Yi, S.H., Kim, D., Kim, C.H., Shim, J.W., Jo, A.Y., Kim, B.W., Lee, H., Lee, S.H., Suh, W., Park, C.H., Koh, H.C., Lee, Y.S., Lanza, R., Kim, K.S., and Lee, S.H. (2011). Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease. J Clin Invest *121*, 2326-35.

Schlaeger, T.M., Daheron, L., Brickler, T.R., Entwisle, S., Chan, K., Cianci, A., DeVine, A., Ettenger, A., Fitzgerald, K., Godfrey, M., Gupta, D., McPherson, J., Malwadkar, P., Gupta, M., Bell, B., Doi, A., Jung, N., Li, X., Lynes, M.S., Brookes, E., Cherry, A.B., Demirbas, D., Tsankov, A.M., Zon, L.I., Rubin, L.L., Feinberg, AP., Meissner, A., Cowan, C.A., and Daley, G.Q. (2015). A comparison of non-integrating reprogramming methods. Nat Biotechnol *33*, 58-63.

Schwarze, S.R., Hruska, K.A., and Dowdy, S.F. (2000). Protein transduction: unrestricted delivery into all cells? Trends Cell Biol *10*, 290-295.

Shi, Y., Desponts, C., Do, J.T., Hahm, H.S., Scholer, H.R and Ding, S. (2008). Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compound. Cell Stem Cell *3*, 568-74.

Singh, A., Upadhyav, V., Upadhyav, A.K., Singh, S.M., and Panda, A.K. (2015). Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. Microb Cell Fact *25*, 14:41

Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G.W., Cook, E.G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., Isacson, O., and Jaenisch, R. (2009). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell *136*, 964-77.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. *126*. 663-676.

Takahashi, K., Okita, K., Nakagawa, M., and Yamanaka, S. (2007). Induction of pluripotent stem cells from fibroblast cultures. Nat Protoc *2*, 3081-3089.

Taranger, C.K., Noer, A., Sorenson, A.L., Hakelien, A.M., Boquest, A.C and Collas, P. (2005). Induction of dedifferentiation, genomewide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Mol Biol Cell *16*, 5719-35.

Their, M., Munst, B., Mielke, S., and Edenhofer, F. (2012). Cellular reprogramming employing recombinant sox2 protein. Stem Cells Int 549846.

Theunissen, T.W., Costa, Y., Radzisheuskaya, A., van Oosten, A.L., Lavial, F., Pain, B., Castro, L.F., and Silva, J.C. (2011). Reprogramming capacity of Nanog is functionally conserved in vertebrates and resides in a unique homeodomain. Development *138*, 4853-65.

Thier, M., Munst, B., and Edenhofer, F. (2010). Exploring refined conditions for reprogramming cells by recombinant Oct4 protein. Int J Dev Biol *54*, 1713-1721.

Vives, E. (2003). Cellular uptake [correction of utake] of the Tat peptide: an endocytosis mechanism following ionic interactions. J Mol Recognit *16*, 265-271.

Vives, E., Brodin, P., and Lebleu, B. (1997). A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem *272*, 16010-16017.

Wadia, J.S., and Dowdy, S.F. (2003). Modulation of cellular function by TAT mediated transduction of full length proteins. Curr Protein Pept Sci *4*, 97-104.

Wang, Y., and Adjaye, J. (2011). A cyclic AMP analog, 8-Br-cAMP, enhances the induction of pluripotency in human fibroblast cells. Stem Cell Rev *7*, 331-341.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., Daley, G.Q., Brack, A.S., Collins, J.J., Cowan, C., Schlaeger, T.M., and Rossi, D.J. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell *7*, 618-630.

Wender, P.A., Mitchell, D.J., Pattabiraman, K., Pelkey, E.T., Steinman, L., and Rothbard, J.B. (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc Natl Acad Sci U S A *97*, 13003-13008.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature *448*, 318-324.

Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Milekovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H.K., and Nagy, A. (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature *458*, 766-770.

Wurm, F.M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol *22*, 1393-1398.

Xu, H., Tsang, K.S., Chan, J.C., Yuan, P., Fan, R., Kaneto, H., and Xu, G. (2013). The combined expression of Pdx1 and MafA with either Ngn3 or NeuroD improves the differentiation efficiency of mouse embryonic stem cells into insulin-producing cells. Cell Transplant *22*, 147-58.

Xu, J., Du, Y., and Deng, H. (2015). Direct lineage reprogramming: strategies, mechanisms, and applications. Cell Stem Cell *16*, 119-34.

Yamanaka, S. (2007). A fresh look at iPS cell. Cell *137*, 13-7.

Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, S., Slukvin, I.I., and Thomson, J.A. (2009). Human induced pluripotent stem cells free of vector and transgene sequences. Science *324*, 797-801.

Zhang, Y., Ma, Y., Gu, J., Liao, B., Li, J., Wong, J., and Jin, Y. (2012). Reprogramming of somatic cells via TAT-mediated protein transduction of recombinant factors. Biomaterials *33*, 5047-5055.

Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., Siuzdak, G., Schöler, H.R., Duan, L., and Ding, S. (2009). Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins. Cell Stem Cell *4*, 381-384.

Figure Caption

List of Figures

Figure 1: The macropinocytosis model of protein transduction. The primary mechanism of protein transduction is an electrostatic interaction between PTD-conjugated macromolecules and the plasma membrane, followed by the penetration into cells by macropinocytosis, and release to the cytoplasm and nuclei by retrograde transport (Noguchi and Matsumoto, 2006a).

Figure 2: Predicted α-helical wheels of original TAT and the synthesization of synthetic TAT peptides. The numbering indicates a sequential amino acid position; values in parentheses indicate the fold change of FITC emission normalized to original TAT peptide (A) (Adapted from Ho *et al.,* 2001).

Figure 3: A comparison of TAT-HIV, TAT33 and TATκ peptides. The blue-coloured letters indicate the replacement of the original amino acid in the original TAT-HIV domain. The green box within the TAT peptide indicates the presence of two Furin sites.

A=Alanine; G=Glysine; K=Lysine; Q=Glutamine; R=Arginine; Y=Tyrosine.

List of tables

Table 1: Amino acid sequence of characterized PTDs.

PTD	Amino acid sequence	Length
HIV-1 TAT	Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg[a]	11 a.a
HSV VP22	Asp-Ala-Ala-Thr-Ala-Thr-Arg-Gly-Arg-Ser-Ala-Ala-Ser-Arg-Pro-Thr-Glu-Arg-Pro-Arg-Ala-Pro-Ala-Arg-Ser-Ala-Ser-Arg-Pro-Arg-Arg-Pro-Val-Glu	34 a.a
Antp	Arg-Gln-Iso-Lys-Iso-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys	16 a.a

Gly-Arg-Lys-Lys-Arg-Arg is a potential nuclear localization sequence.

Ala-Alanine, Tyr-Tyrosine, Gly-Glycine, Arg-Arginine, Lys-Lysine, Gln-Glutamine, Asp-Aspartic acid, Thr-Threonine, Ser-Serine, Pro-Proline, Phe-Phenylalanine, Trp-Trytophan, Met-Methionine.

Table 2: A wide variety of macromolecules have been covalently fused to PTDs.

Macromolecules	***PTD-conjugated***	***Target cells***	***references***
Protein/peptide	TAT-β-galactosidase	Rat salivary gland cell lines, organ culture, rat submandibular gland (*in vivo*)	(Barka *et al.*, 2000)
		Murine HSCs	

		Murine HSCs	
	TAT-HoxB4		(Krosl *et al.*, 2003)
	TAT-Zfx		(Xu *et al.*, 2009)
Antisense oligonucleotide	A short peptide vector/oligonucleotide complexes (MPG)	Human fibroblast HS68 and NIH3T3 cell lines	(Morris *et al.*, 1997)
Peptide nucleic acid (PNA)	Phosphotyrosine phosphatases (PTPases)	Pancreatic islets from rat	(Ostenson *et al.*, 2002)
Iron beads/ nanoparticles	TAT-CLIO Super paramagnetic iron oxide (CLIO)	Murine T-cells	(Dodd *et al.*, 2001)
Liposomes			
	large drug carriers, 200nm liposome-TAT	BT20 (human breast tumour), Lewis lung carcinoma (LLC, mouse lung carcinoma) and H9C2 (rat embryonic cardiac myocytes)	(Torchilin *et al.*, 2001)